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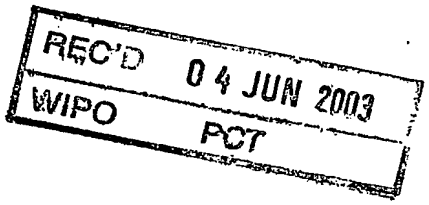

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Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

02290610.1

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For the President of the European Patent Office

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R C van Dijk

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
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Mutated gene coding for a lat protein and the biological applications thereof.

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
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C12N15/00

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Mutated gene coding for a LAT protein and the biological applications thereof.

The present invention relates to a mutated gene coding for a mutant LAT protein.

The invention further relates to biological structures containing said mutant, particularly, non-human gene mutated animals, plasmids, chromosomal DNAs, embryos comprising said mutated gene, and applications thereof.

Background Art

A key event in the parthenogenesis is the production of antibodies of the IgE class. Hypergammaglobulinemia E results from loss of immunoregulation. More specifically, T lymphocyte abnormalities have been reported in a number of pathologic hyper IgE conditions and are the object of much research aiming at developing pharmaceutical compounds that will prevent atopic allergy and asthma.

TCR recognize peptide fragments bound to major histocompatibility complex (MHC) molecules and relay this information to the interior of the T cell via adapter proteins. One of these, the adapter LAT (Linker for Activation of T cells), coordinates the assembly of signaling complexes through multiple tyrosine residues within its intracytoplasmic segment. Upon TCR-induced phosphorylation, each of these tyrosines manifests some specialization in the signaling proteins it recruits. For instance, mutation of tyrosine 136 (Y136) selectively eliminates binding of phospholipase Cγ1 (PLC-γ1) whereas the simultaneous mutation of Y175, Y195 and Y235 results in loss of binding of downstream adapters Gads and Grb-2.

The inventors provided genetic evidence that LAT exerts an unanticipated inhibitory function on the differentiation of CD4 helper T (T_H) cells into T_H2 cells. Mice homozygous for a selectively mutation of a single LAT tyrosine (LAT Y136F) show both an impeded T cell development and a precocious and spontaneous accumulation of polyclonal T_H2 cells which chronically produce large amounts of interleukin 4, 5, 10 and 13. This exaggerated T_H2 differentiation leads in turn to tissue eosinophilia and to the maturation of massive numbers of plasma cells secreting IgE and IgG1 antibodies (see Figure 1). Thus, in addition to known positive signaling, LAT appears also essential for establishing inhibitory signals control T cell homeostasis.

Description of drawings

Figure 1 is a diagram disclosing the immune system development of mutant mice.

Figure 2 illustrates the LAT Y136F knock-in strategy:

(1) : the partial restriction map of the wild-type LAT gene.

(2) : the targeting vector used for the introduction of the LAT Y136F mutation.

(3) : the structure of the targeted allele following homologous recombination.

(4) : the final structure of the targeted allele after removal of the neo^r gene *via* Cre-mediated recombination.

Figure 3 illustrates the aberrant growth of lymphoid organs in the mice : thymus (A), spleen (B) and lymph nodes (C).

Figure 4 relates to constitutive type-2 cytokine production in CD4 T cells freshly isolated from LAT^{Y136F} peripheral lymphoid organs.

Figure 5 relates to a phenotypic analysis of T cells from wild-type and LAT^{Y136F} mice.

Figure 6 illustrates eosinophilia in 6 weeks old LATY136F lymphoid organs.

A: Dot plot panel showing the gate selected for the analysis described in panel B and for the sorted cells picture in panel C.

B: Single color histograms of gated cells labelled with antibodies characterizing eosinophils.

C: Hematoxylin and eosin staining of sorted cells.

Figure 7 illustrates the hyperactivity of B lymphocytes: massive serum levels of IgE and IgG1 antibodies in unimmunized LAT^{Y136F} mice.

Description

The object of the present invention is to provide a mutated gene coding for a mutant LAT protein, the sequence of which corresponds to the wild type sequence and contains a single mutation of the tyrosine at position 136.

Preferably, the LAT mutant protein sequence contains the single mutation Y136F.

In this application, LAT Y136F will refer to mutation itself contrary to LAT^{Y136F} which will refer to mutants, mice or products derived from this mutation.

This single mutation is able to induce the development of pathologies associated with exacerbated T_H2 immunity. Characteristics of the phenotype associated with this mutation are described in the following examples.

In a preferred embodiment, the sequence of the gene encoding LAT protein corresponds to sequence ID N°1.

A further object of the invention is to provide non-human gene-mutated animals having the mutated LAT gene of the invention.

In particular, the germs cells and somatic cells of the animals, contain the mutated LAT gene as claimed in any of claims 1 to 3, as a result of chromosomal incorporation into the animal genome, or into an ancestor of said animal.

Preferably, the animals according to the invention are mammals, and in particular, they are rodents.

The magnified and accelerated sequence of pathological events observed in the LAT Y136F mice permits to readily start tests and studies. For example, mutant mice phenotype is achieved when they are 4 weeks old.

The present invention also encompasses plasmids comprising a DNA or a part thereof, having a sequence corresponding to the mutated gene according to the invention.

In a preferred embodiment, the plasmids of the invention contain a restriction enzyme cleavage site, which is introduced in the intron 3' of exon 7.

Advantageously, the restriction enzyme cleavage site is a Bgl II restriction site.

The invention further includes chromosomal DNAs containing exon 7 of the mutated gene (SEQ ID N°2).

Then, the mutated LAT protein contains the mutated amino acid sequence of exon 7 (SEQ ID N°3).

Consequently, the invention also encompasses embryos introduced with the plasmids of the invention, and embryos obtained by homologous recombination using the plasmids of the invention.

In a preferred embodiment, the embryos are embryonic stem cells derived from a mouse. Advantageously, the ES cells are CK35 129/SV ES cells.

The invention also provide oligonucleotides, the sequence of which corresponds to SEQ ID N°4 and/or SEQ ID N°5 as probes to screen the presence of the mutation.

The mutant mice are useful in various applications of interest, in particular:

- to analyze the impact of drugs on the molecular mechanisms that lead to exacerbated IgE production as well as tissue eosinophilia, and
- as a bioreactor allowing the dedicated production of IgE antibody of known specificity prior to or following a step of humanization of the LAT^{Y136F} mouse.

Consequently, the present invention provides models of allergy and/or asthma disease comprising animals according to the invention.

In particular, the animals of the invention can be used as models of eosinophilia.

Due to the increased sensitivity of population, health difficulties such as asthma or allergies are more frequent. The animals according to the invention are suitable models to help the research in these domains.

Accordingly, the present invention provides a method of

screening for a drug for treatment of allergy and/or asthma disease comprising the step of subjecting the animals according to the invention, which are administered with the drug to a comparison with said animals, not administered with the drug.

In still another application, the present invention provides bioreactors for a large-scale production of human IgE antibodies comprising the animals according to the invention.

Really, LAT^{Y136F} mice are able to produce tremendous amount of IgE, as it is described in example 2. IgE produced by mutant mice are useful for applications such as desensitization.

Other characteristics and advantages of the invention are given in the following examples with reference to figures 2 to 7.

Example 1: Production of mutant mice

To test *in vivo* the importance of LAT^{Y136}, the inventors generated knock-in mice with a mutation replacing Y136 with phenylalanine (Y136F).

1. Materials and methods

Mice

Mice were maintained in a specific pathogen-free animal facility.

LAT^{Y136F} mutation.

LAT genomic clones were isolated from a 129/Ola phage library. After establishing the nucleotide sequence and

the exon-intron structure of the LAT gene, the tyrosine residue found at position 136 and encoded by exon 7 was mutated to phenylalanine. Mutagenesis was performed on a 1717-bp Eco RI-Xba I fragment encompassing part of exon 5, exons 6, 7 and 8. In addition to the intended mutation, a new Bgl II restriction enzyme cleavage site was introduced in the intron 3' of exon 7 to accommodate the LoxP-flanked neo^r gene and facilitate subsequent identification of LAT^{Y136F} mutant mice. Finally, the targeting construct was extended to give 1.7 kb and 4.8 kb of homologous sequences 5' and 3' of the EcoRI-XbaI fragment, respectively (see Fig. 2). After electroporation of CK35 129/SV ES cells (C. Kress *et al.*, 1998), and selection in G418, colonies were screened for homologous recombination by Southern blot analysis. The 5' single-copy probe is a 0.9-kb Bgl II-Xba I fragment isolated from a LAT genomic clone. When tested on Bgl II-digested DNA, the 5' probe hybridizes either to a 8.5 kb wild-type fragment or to a 4.5 kb recombinant fragment. Homologous recombination events at the 3' side were screened by long range PCR. Homologous recombinant ES clones were further checked for the presence of the intended mutation by sequencing the genomic segment corresponding to exon 7. Finally, a neo probe was used to ensure that adventitious non homologous recombination events had not occurred in the selected clones.

Production of mutant mice.

Mutant ES cells were injected into Balb/c blastocysts. Two LAT^{Y136F} recombinant ES cell clones were found capable of germ line transmission. The two mutant mouse lines were first bred to Deleter mice (Schwenk. F *et al.*, 1995) to eliminate the Lox P-flanked neomycin cassette, and intercrossed to produce homozygous mutant mice. The two independently-derived mutant lines showed indistinguishable phenotype. To confirm that the LAT Y136F

mutation had been genuinely introduced, LAT transcripts were cloned by reverse transcription and PCR amplification from the thymus of the mutated mice, and the presence of the intended mutation confirmed by DNA sequence analysis. Screening of mice for the presence of the LAT Y136F mutation was performed by PCR using the following pairs of oligonucleotides:

c : 5'-GTGGCAAGCTACGAGAACCAGGGT-3' (SEQ ID N°4);

d : 5'-GACGAAGGAGCAAAGGTGGAAGGA-3' (SEQ ID N°5).

The single Lox P site remaining in the LAT Y136F allele after deletion of the neo^r resulted in an amplified PCR product 140 bp-longer than the 510 bp-long fragment amplified from the wild-type LAT allele.

2) Mutant mice development

Mice homozygous for the LAT^{Y136F} mutation, hereafter denoted LAT^{Y136F}, were born at expected Mendelian frequencies and their T cells contained levels of LAT proteins similar to wild-type T cells. At birth LAT^{Y136F} mice displayed peripheral lymphoid organs of normal size. Beginning at about 3 weeks, however, the spleen and lymph nodes of the mutant mice started to enlarge relative to wild-type littermates, such that by 15 weeks of age, spleen cellularity was approximately 10 times that of wild-type mice (Fig.3 A-C). Despite marked lymphocytic infiltrations in the lung, liver and kidney, homozygotes lived to at least 17 weeks of age, and no chronic intestinal inflammation or tumor formation was observed. The effects of the LATY136F mutation were only detectable after breeding mice to homozygosity or to mice carrying a null allele of the LAT gene.

Example 2: Effect of the mutation: spontaneous exaggerated T helper type 2 immunity in mice

1. Materials and methods

Purification of CD4+ T cells and eosinophils.

Lymph node and spleen cells from several mice were pooled and the CD4+ cells purified using a high gradient magnetic cell separation system (S. Miltenyi et al., 1990). Eosinophils were sorted on a FACSvantage™ on the basis of their FSChigh, HSA+, and CD11b+ phenotype.

Antibodies and flow cytometric analysis.

Before staining, cells were preincubated on ice for at least 10 min with polyclonal mouse and rat Ig to block Fc receptors. Flow cytometric analysis was performed as described previously (M. Malissen *et al.*, 1995). All the antibodies were from BD PharMingen except the anti-CCR3 antibody that was purchased from R&D.

Staining for intracellular cytokines.

Before intracellular cytokine staining, cells (1.5×10^6) were cultured for 4 h in the presence of monensin (GolgiStop; BD PharMingen) at a final concentration of 2 μM. Cells were then immediately placed on ice, washed, resuspended in PBS 1X, 1% FCS, 0.20% sodium azide, and stained with an APC-conjugated anti-CD4 antibody. For intracellular cytokine staining, cells were first fixed using the cytofix/cytoperm kit (BD PharMingen). Each cell sample was subsequently split into aliquots that were separately stained with (1) a combination of FITC-conjugated anti-IFN-α and PE-conjugated anti-IL-2 antibodies, (2) a combination of FITC-conjugated anti-IL-5 and PE-conjugated anti-IL-4 antibodies, and (3) a combination of fluorochrome-conjugated and isotype-matched negative control Ig (BD PharMingen). After a final wash, CD4+ cells (10^4) were analyzed on a FACSCalibur™ flow cytometer after gating out dead cells

using forward and side scatters.

RNase protection assay.

For multiplex cytokine transcript analysis, total cellular RNA was isolated from the specified cells using TRIzol (GIBCO-BRL Life Technologies) and analyzed by ribonuclease protection assay using an MCK-1 RiboQuant™ custom mouse template set (BD Pharmingen). Briefly, ^{32}P-labeled riboprobes were mixed with 10 μg of RNA, incubated at 56°C for 12 to 16 hours, and then treated with a mixture of RNases A and T1 and proteinase K. RNase-protected ^{32}P-labeled RNA fragments were separated on denaturing polyacrylamide gels and the intensity of the bands evaluated with a Fuji imaging plate system.

Determination of serum isotype-specific immunoglobulin levels.

The titres of polyclonal IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA antibodies and κ and λ light chains were determined using isotype-specific ELISA (Southern Biotechnology). The concentrations of IgG1 and IgE were determined by comparing test sample dilution series values with isotype control standards.

2. Results

A prominent phenotype of the CD4 T cells found in LAT^{Y136F} mice was revealed when the inventors measured their ability to make cytokines. Due to the short half-lives of cytokines and of their transcripts, their analysis generally requires restimulation of T cells in vitro with PMA and ionomycin. A multiprobe RNase protection assay detecting levels of transcripts of 9 cytokines showed that CD4 T cells freshly isolated from LAT^{Y136F} mice contained sufficient IL-4 and IL-10 transcripts to allow their detection even without *ex vivo* restimulation (Fig.

4A). Upon activation by PMA/ionomycin the levels of IL-4 and IL-10 transcripts they contained were further increased, and IL-5, IL-13, and IFN-α transcripts became readily detectable (Fig. 4B). In marked contrast, wild-type CD4 T cells yielded only the IL-2 and IFN-α transcripts expected for primary T cells. Analysis of IL-4 production at the single cell level, showed that following a 4 hr activation with PMA/ionomycin, close to 80% of the CD4 T cells isolated from LAT^{Y136F} mice expressed very high levels of IL-4 (Fig. 4C). Consistent with the notion that these CD4 T cells were refractory to TCR stimuli, none of them scored as IL-4+ in response to TCR cross-linking (Fig. 4C). Thus, LAT Y136F spontaneously developed a high frequency of T_H2 cells. In the case of wild-type CD4 T cells, T_H2 polarization of such magnitude is only achieved following prolonged antigenic stimulation in the presence of IL-4.

Light scatter analysis of thymic and lymph node cells from LAT^{Y136F} mice older than 4 weeks revealed a unique cell population that was almost absent from age-matched wild-type mice, and showed both an intermediate forward scatter and a high side scatter (Fig. 5A, 5B, 6A). Based on several of criteria, these cells were identified as eosinophils (Fig. 6). Minute numbers of eosinophils normally reside in wild-type thymi, and their augmentation in LAT^{Y136F} thymi may primarily result from an intrinsic expression of LAT^{Y136F} molecules. However, LAT transcripts were undetectable in eosinophils purified from LAT^{Y136F} mice, meaning that the thymic and lymph node eosinophilia they manifest result from the production of IL-5 by the abnormal CD4 cells present in these mutant mice.

Secondary lymphoid organs of 6-week old LAT^{Y136F} mice contained 7 to 10 times more B cells than their wild-type

counterparts. Thus, the splenomegaly and generalized lymphadenopathy that developed in young LAT^{Y136F} mice can be mostly accounted for by cells belonging to the T and B cell lineages. Over 90% of the mature B cells found in the spleen and lymph nodes of 6-week old wild type littermates had a resting phenotype (Fig. 7A). In marked contrast, only 25% of the B cells found in the enlarged secondary lymphoid organs of age-matched LAT^{Y136F} littermates showed a resting phenotype. Among the remaining B cells, 25% showed an hyperactivated phenotype, and 50% expressed a phenotype typical of antibody producing cells. Coincident with the presence of these latter cells, serum IgG1 concentrations were elevated approximately 200 times compared to wild-type mice, whereas those of IgE were elevated 2500 to 10000 times (Fig. 7C). In contrast, the levels of the other Ig isotypes did not differ significantly from those of wild-type serum (Fig. 7B). In support of a polyclonal hypergammaglobulinemia G1 and E, the concentrations of kappa and lambda light chains were both markedly augmented in the serum of LAT^{Y136F} mice (Fig. 7B). Notably, IgE and IgG1 antibody concentrations reached a plateau as early as 5 weeks of age (Fig. 7C), the values of which exceeded the extraordinarily large amounts of IgE and IgG1 previously reported for mice deprived of NFATc2 and NFATc3 transcription factors. Given that B cells do not express LAT proteins, and considering that isotype switching to IgE and IgG1 is highly dependent on the presence of IL-4 and IL-13, the overproduction of IgE and IgG1 noted in LAT^{Y136F} mice is secondary to the presence of an abnormally high frequency of T_H2 effectors.

Example 3: Production of IgE

Mice expressing humanized IgE are developed by conventional knock-in strategy in which the genetic

segment corresponding to the constant exons of the IgE gene is substituted by the corresponding human sequence. Mice with a humanized IgE locus are bred into LAT Y136F mice. Following immunization, B cell hybridomas producing specific human IgE are produced, and the resulting specific human IgE are used as "standard" in clinical assays aiming at characterizing atopic allergens present in patients."

Example 4: Screening for a drug

Mutant mice and control ones will be treated with a variety of drugs or original compounds. Their effects will be analyzed in vivo by measuring various parameters such as:

- T_H2 cells differentiation.
- Production of T_H2 types cytokines
- Eosinophilia
- Hypergammaglobulinemia G1 and E .

References

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Malissen, M., Gillet, A., Ardouin, L., Bouvier, G., Trucy, J., Ferrier, P., Vivier, E., and Malissen, B. (1995). Altered T cell development in mice with a targeted mutation of the CD3- epsilon gene, Embo J *14*, 4641-53.

Miltenyi, S., Muller, W., Weichel, W., and Radbruch, A. (1990). High gradient magnetic cell separation with MACS, Cytometry *11*, 231-8.

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Claims

1. A mutated gene coding for a mutant LAT protein, the sequence of which corresponds to the wild type sequence and contains a single mutation of the tyrosine at position 136.

2. A mutated gene as claimed in claim 1, the LAT mutant protein sequence of which contains the single mutation Y136F.

3. A mutated gene as claimed in claim 1 or 2, the sequence of which corresponds to sequence ID N°1.

4. A non-human gene-mutated animal having a mutated LAT gene as claimed in any of claims 1 to 3.

5. A non-human gene mutated animal whose germs cells and somatic cells contain the mutated LAT gene as claimed in any of claims 1 to 3, as a result of chromosomal incorporation into the animal genome, or into an ancestor of said animal.

6. An animal as claimed in claim 4 or 5, which is a mammal.

7. An animal as claimed in any of claims 4 to 6, which is a rodent.

8. A plasmid comprising a DNA or a part thereof, having a sequence corresponding to the mutated gene as claimed in any of claims 1 to 3.

9. A plasmid according to claim 8, wherein a restriction enzyme cleavage site is introduced in the intron 3' of

exon 7.

10. A plasmid according to claim 9, wherein a restriction enzyme cleavage site is a Bgl II restriction site.

11. A chromosomal DNA containing exon 7 of the mutated gene (SEQ ID N°2).

12. An embryo introduced with the plasmid according to claim 8 or 10.

13. An embryo obtained by homologous recombination using the plasmid according to claim 8 or 10.

14. An embryo according to claim 12 or 13, wherein the embryo consists of embryonic stem cells derived from a mouse.

15. An embryo according to claim 14, wherein ES cell are CK35 129/SV ES cell.

16. An oligonucleotide, the sequence of which corresponds to SEQ ID N°4 and/or SEQ ID N°5, as a probe.

17. A model of allergy and/or asthma disease comprising an animal according to any of claims 4 to 7.

18. A model of eosinophilia comprising an animal according to any of claims 4 to 7.

19. A method of screening for a drug for treatment of allergy and/or asthma disease comprising the step of subjecting the gene-mutated animals according to any of claims 4 to 7 which are administered with the drug to a comparison with said gene-mutated animals, not

administered with the drug.

20. A bioreactor for a large scale production of human IgE antibodies comprising an animal according to claims 4 to 7.

Abstract

The present invention relates to a mutated gene coding for a mutant LAT protein, the sequence of which corresponds to the wild type sequence and contains a single mutation of the tyrosine at position 136.

The invention further relates to biological structures containing said mutant, particularly, non-human gene mutated animals, plasmids, chromosomal DNAs, embryos comprising said mutated gene, and applications thereof.

Figure 1

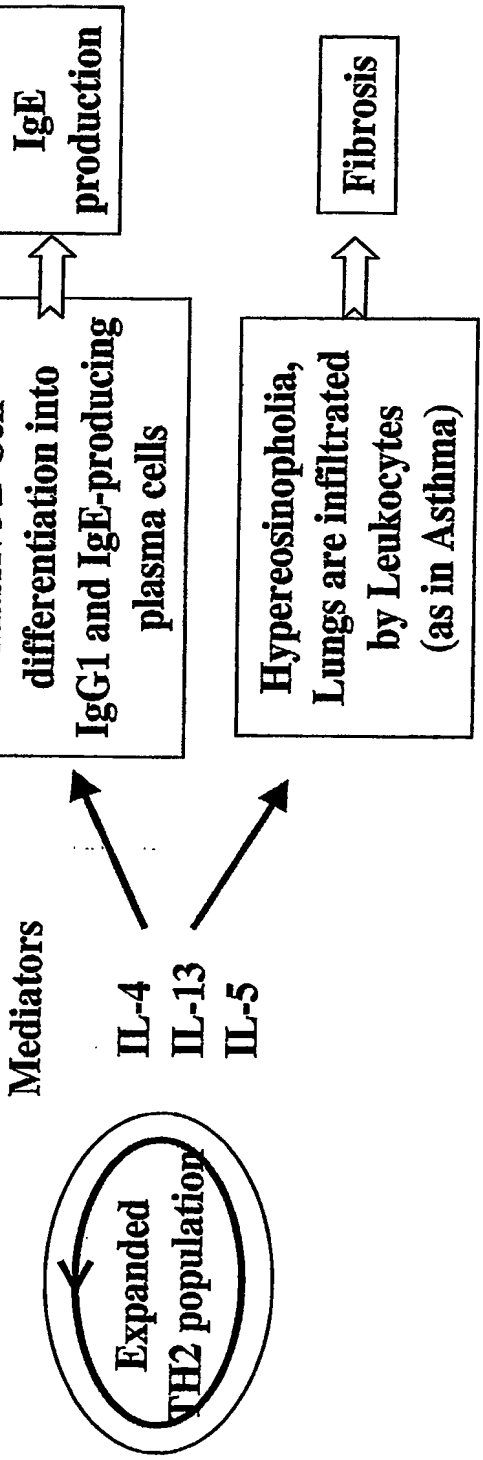

Expanded TH2 population
Mediators
IL-4
IL-13
IL-5
Massive B Cell differentiation into IgG1 and IgE-producing plasma cells
Hypereosinopholia, Lungs are infiltrated by Leukocytes (as in Asthma)
IgE production
Fibrosis

Figure 2

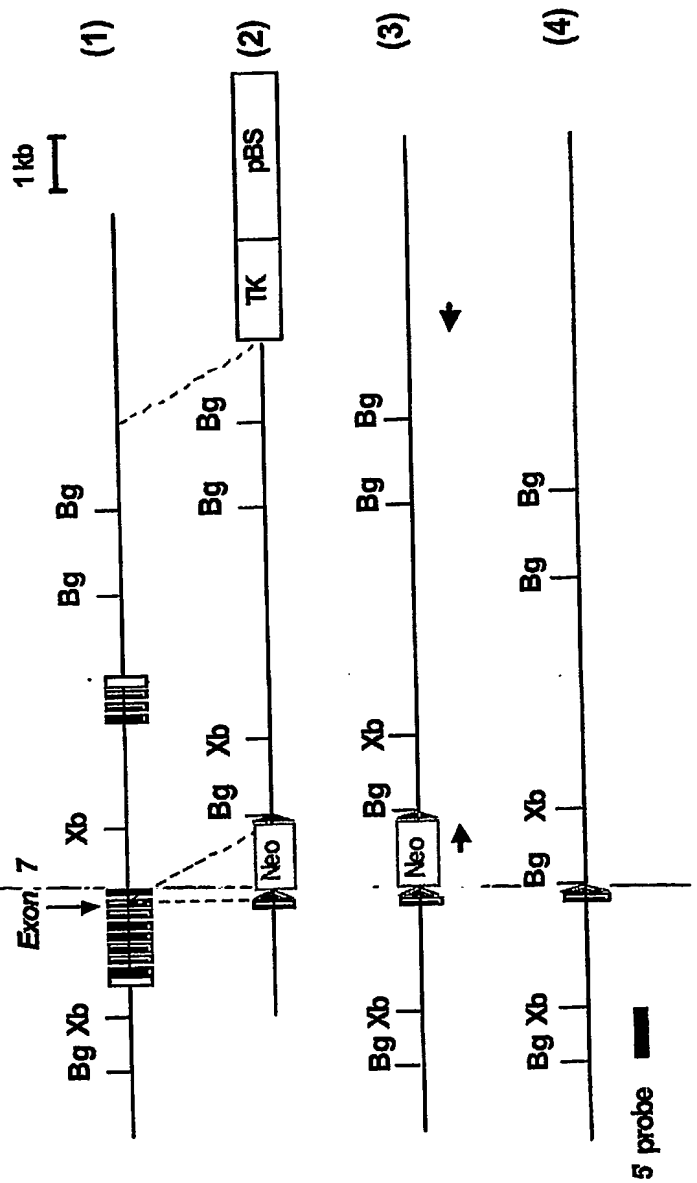


Figure 3

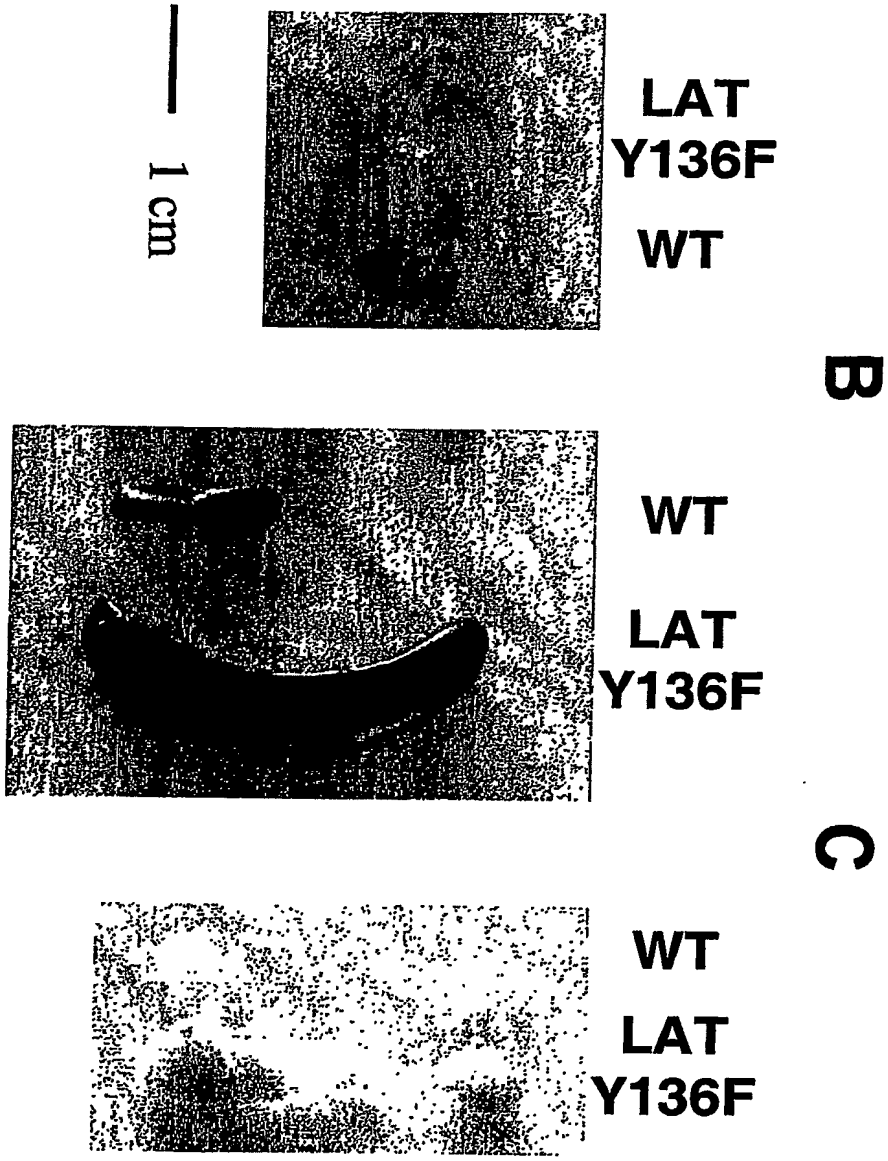
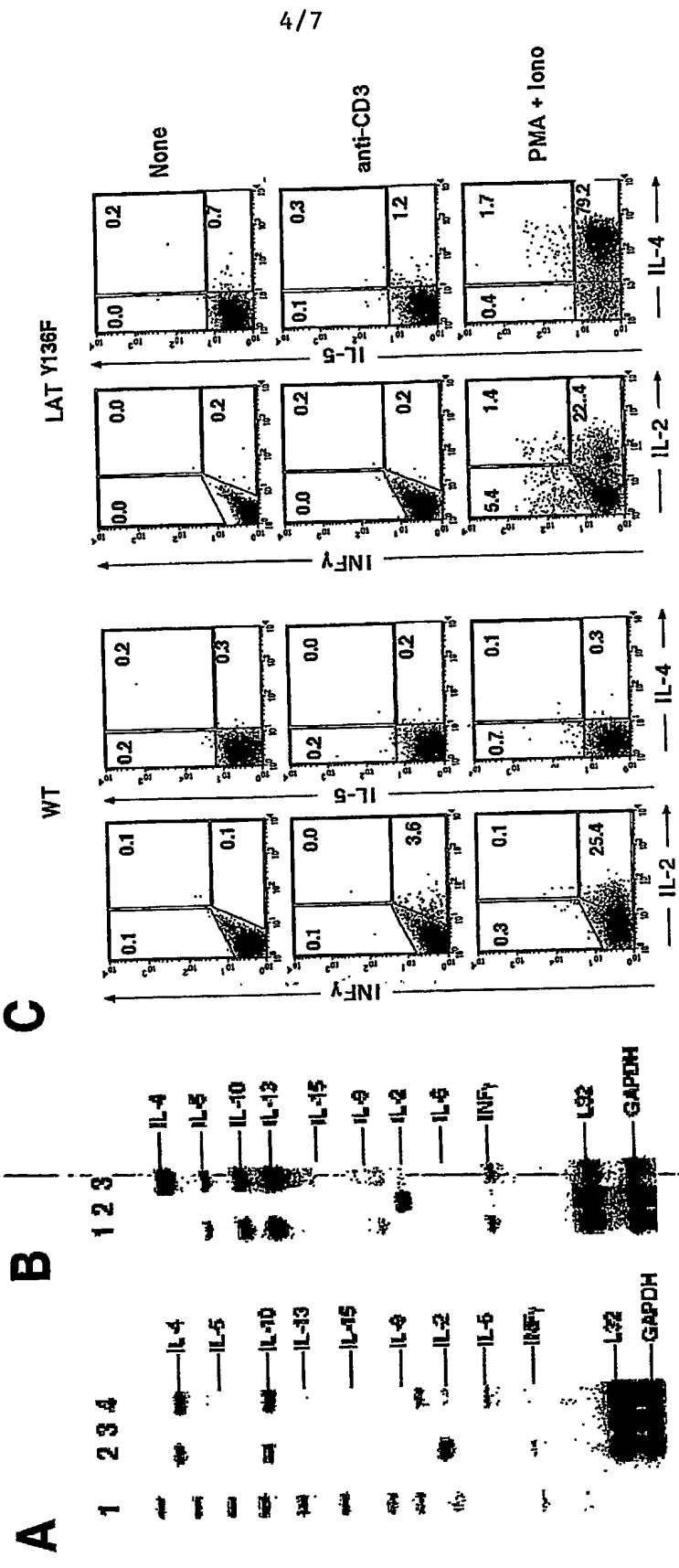


Figure 4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IL-6
INFγ
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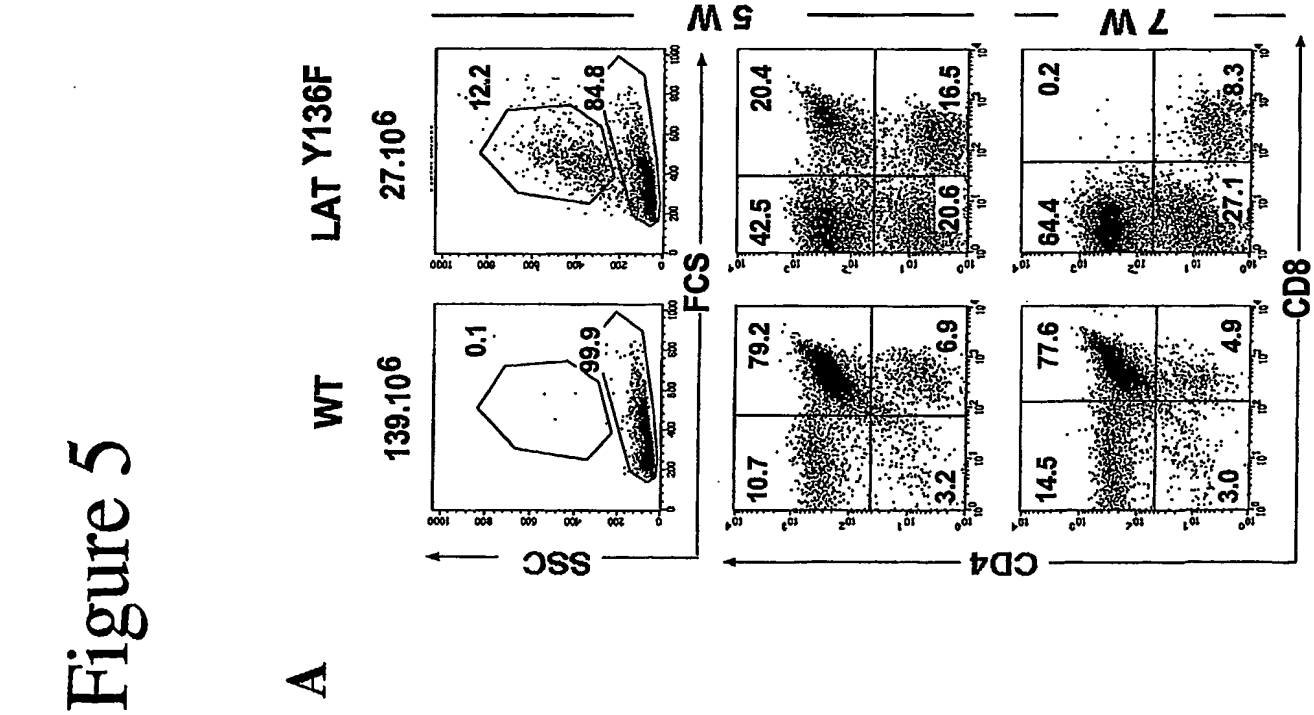
Figure 5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3.2
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20.4
20.6
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14.5
77.6
3.0
4.9
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27.1
8.3
5 W
7 W
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Figure 6

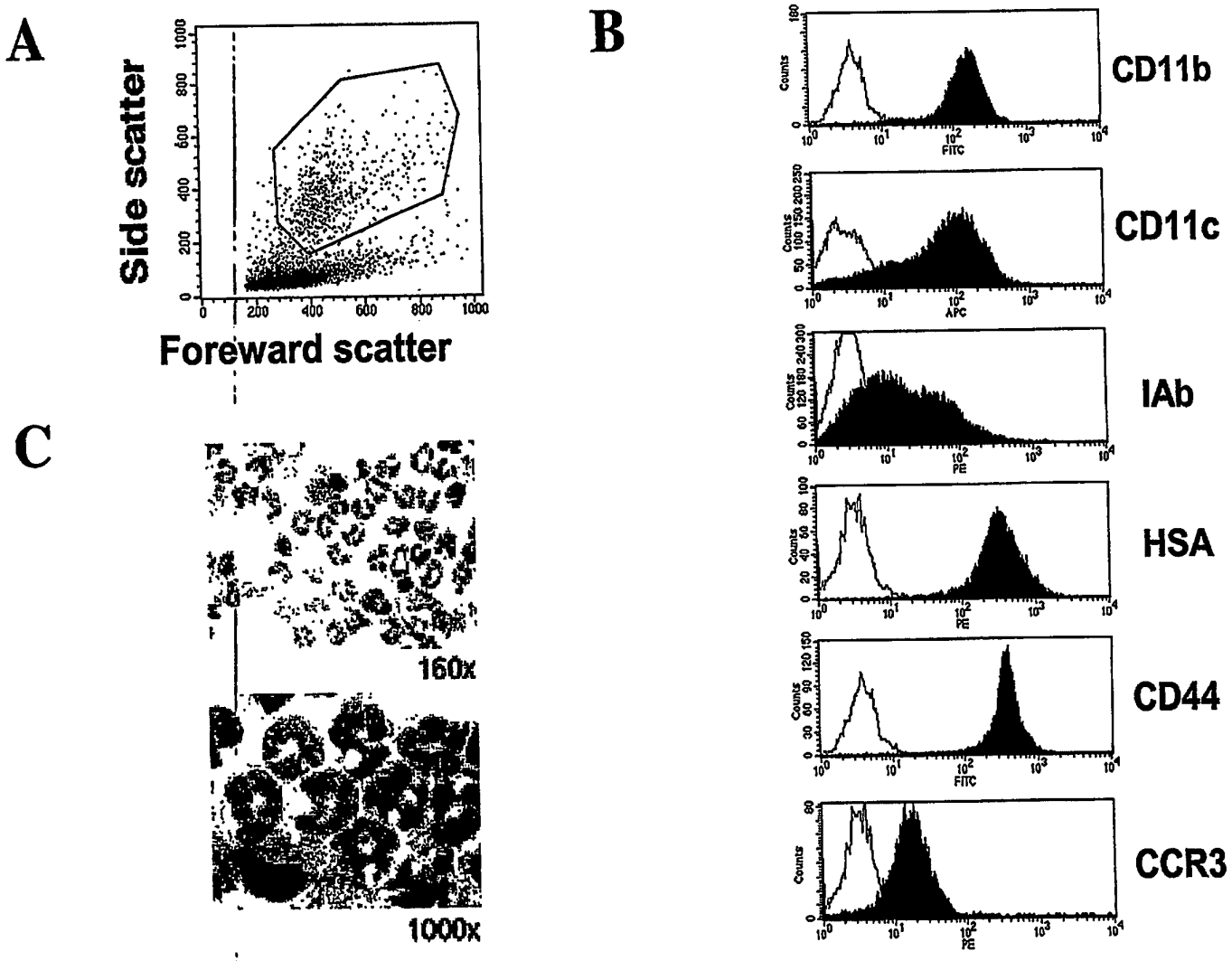
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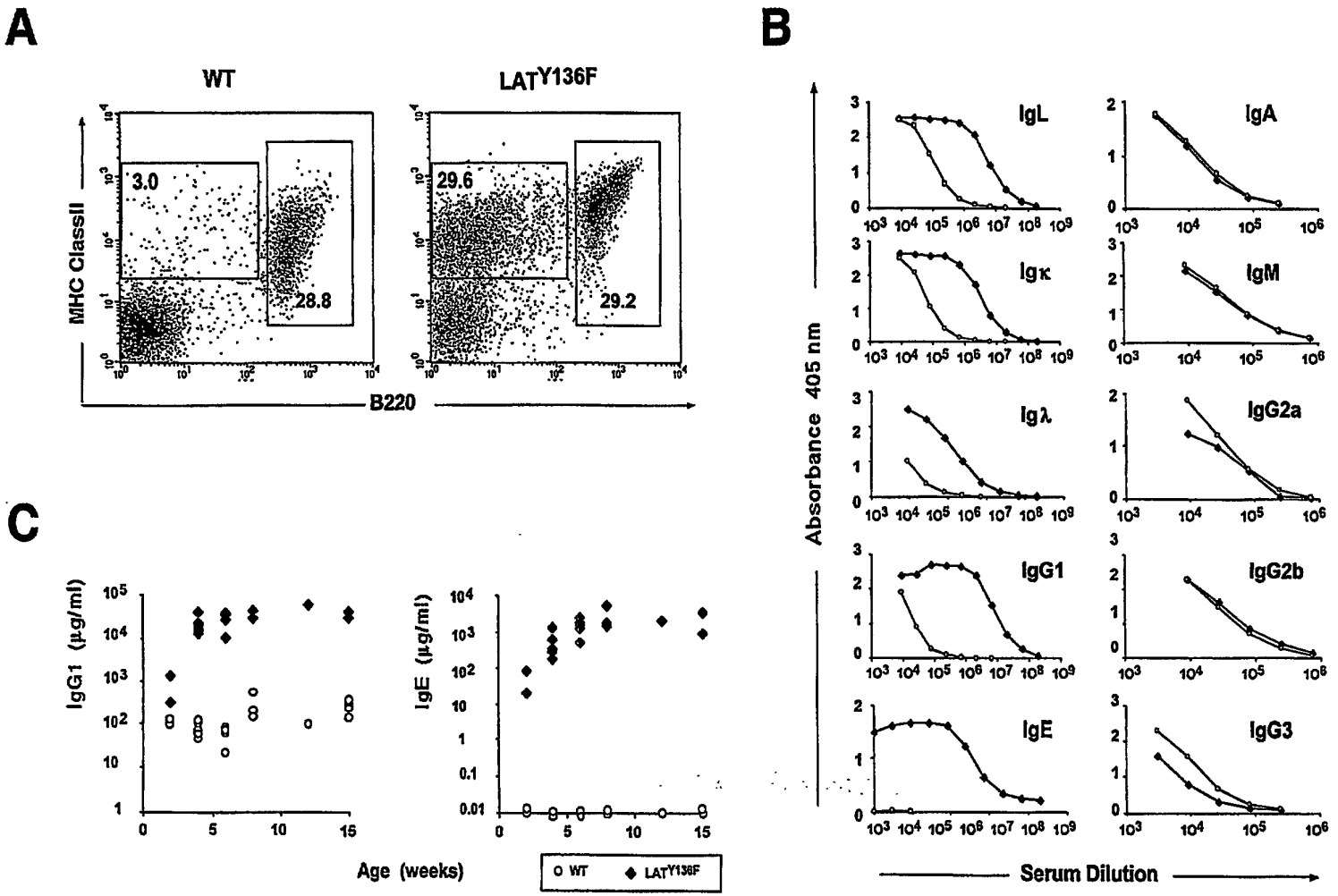
Figure 7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IgG1
IgG2b
IgE
IgG3
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C
IgG1 (µg/ml)
IgE (µg/ml)
Age (weeks)
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LATY136F

Sequences

SEQ ID N°1

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